Day 1 (Note: All the following equipments and reagents come from the Fromme Lab)

1. Transform expression vector into desired chassis. This step is optional if glycerol stock is available.

Day 2 (2 hours)

1. Inoculate cells into 25 mL Terrific Broth (TB) + phosphate buffer. Grow overnight shaking in 37 C.
2. Prepare 900 mL TB and appropriate amount of phosphate buffer for inoculation.

Day 3 (at least 2 hours)

1. Add 100 mL phosphate buffer to 900 mL TB. Add appropriate amount of antibiotics
2. Inoculate 25 mL overnight culture into 1 L TB medium.
3. Grow shaking at 37 C. Monitor OD (check by 1:10 dilution) until it reaches at least 3 (ideally 4). This usually takes 7 hours, but may take 12+ hours.
4. Once desired OD is attained, reduce temperature down to 15 C and continue shaking for around 1 hour.
5. Induce with appropriate inducer. Allow induction to occur overnight while shaking at 15 C.

Day 4 Part 1 (At least 5 hours for 4 samples. If you exceed a multiple of 4, add 1 extra hour)

1. Spin down 1 L cultures at 4 C (5000 rpm for 10 minutes is sufficient).
2. Decant media. Resuspend cells in 25 mL ice cold lysis buffer with EDTA-free Roche protease inhibitor tablet (1 tablet per 200 mL is sufficient). I generally like to keep an extra 50 mL for downstream procedures.
    a. Lysis Buffer: Tris pH 8.0 + Glycerol + Imidazole + NaCl + H2O
3. Add phenylmethanesulfonylfluoride (PMSF) and beta-mercaptoethanol (BME) to render final concentration about 1 mM each. Does not have to be super precise.
4. Sonicate samples at 100% power. Usually, I do 15 pulses with 1 second pauses between pulses. I sonicate each sample 3 times and allow them to cool on ice for 5 minutes between each sonication.
5. Spin down lysates 10,000 rpm for 30 minutes (fixed angle rotor)
6. Separate supernatant and pellet. Take a gel sample of the pellet after redissolving in water. Take a gel sample of the supernatant.
    a. For iGEM, I chose not to take pellet gel samples. If my 160 kDa proteins can stay in the supernatant, most proteins we work with will probably stay as well.
7. Add 250 uL Ni-NTA agarose beads to supernatant. Allow sample to rotate in 4 C for 1 hour. Meanwhile, clean up the crazy amount of mess.
8. Precipitate beads at 4C 1500 rpm for 3 minutes (swinging bucket rotor)
9. Carefully separate the “unbound” supernatant and the beads.

10. Transfer beads to eppendorf for easier handling. Use a bit of of lysis buffer to help with the transfer.
11. Wash the beads with 1 mL lysis buffer 3 times. Spin down at 4 C 1500 rpm for 3 minutes. Decant supernatant into unbound. Take gel samples of unbound after all washes.
12. Add elution buffer to beads. Allow to incubate at room temperature for 3-5 minutes before spinning the beads down. This time, save the supernatant. Repeat two more times.
    a. Elution Buffer = 10 mL Lysis Buffer + 480 uL 5M Imidazole
    b. For expression tests, 200 uL per elution is sufficient.
13. Take gel samples of the eluate.

At this point, you can flash freeze all your eluates and gel samples with liquid nitrogen and store at -80 C if necessary. Note that freeze thaw cycles easily degrade proteins.

Day 4 Part 2 (at least 4 hours)

1. Run gel samples on appropriate SDS-PAGE gels. It may be worth running two sets of gels: one for coomassie staining and one for western blotting
    a. We tend to run 25 mA 250 V constant amps for 50-55 minutes. Nothing seems to work for iGEM unfortunately
    b. IMPORTANT: For Western blots, use prestained ladder. These will be visible on the membrane after transfer.
2. Before gel finishes running (reserve 20 minutes), get an appropriately-sized PVDF membrane. Soak it in methanol for 5 minutes, then soak it in water for another 5 minutes. Finally, equilibrate in PVDF buffer for another 5 minutes.
3. When gels finish running, take one set and stain in coomassie for 20 minutes. Reuse coomassie. Afterwards, destain in 10% acetic acid for half an hour.
    a. At this point, you should be able to detect your proteins if it expressed well. If so, congrats!. Wash the gels and destain overnight in water to take a picture tomorrow. No need for western blots. If not...well, it's science, deal with it.
4. For the other gels, set up into western blot "sandwich."
    a. The order of the sandwich changes depending on the equipment and direction of current. But in general, there should be two pieces of chromatography paper with the gel and membrane stuck between.
    b. The chromatography paper should be soaked in PVDF membrane, and use a tube or marker to ensure no bubbles in the sandwich
5. Transfer at 4 C for 2 hours, with stir bar spinning at 80-100 rpm.
    a. The exact settings vary depending on protein. I did 60 V at 3.0 mA, constant voltage
    b. It is possible to over-transfer, resulting in proteins on your chromatography paper. Be careful (although most of the time this shouldn't happen).
    c. If current is < 0.7 mA at the end of transfer, reuse PVDF buffer.

6. Block the membrane in 4% milk in PBST for half an hour
    a. PBST = PBS + 0.02% Tween-20. 25 mL is sufficient.
7. Rinse once with PBST.Incubate with primary antibodies overnight at 4 C on rocking platform.
    a. For us, the primary antibody is mouse anti-His

Day 5 (3 hours)

1. SAVE AND REUSE THE PRIMARY ANTIBODIES
2. Do 3*5 minute washes in PBST.
3. Meanwhile, prepare secondary antibody in 2% milk in PBST. After the washes, incubate the membrane in secondary antibody at room temperature for 1 hour.
    a. Ideally, use a tray that is not transparent.
    b. Concentration varies, but 1:5000 seems reasonable (5 uL in 25 mL)
4. After that, dump the secondary antibodies. Do at least 3*10 minute washes in PBST.
5. Mix horseradish peroxidase solution and ECL blotting substrate solution in a 1:1 ratio (4 mL is good). Allow to warm up at room temperature.
    a. However, do this after you're close to done with the last wash. This solution is light sensitive.
6. After the last wash, place membrane on a piece of plastic wrapping, closer to one end (the plastic wrap will be folded over). Dry off remnant PBST with paper towel. Apply ECL solution to membrane uniformly and incubate for 5 minutes.
    a. This is time-sensitive. Don't go over 5 minutes or you'll regret it.
7. Dry off ECL solution with paper towel. Fold plastic wrapping over the membrane as smoothly as possible. Use razor to resize as needed.
8. Tape the membrane and plastic wrap to a film case. Bring scissor and blotting film to developing room.
9. Inside the developing room, turn on only the super dim red lights. Take out blotting films and resize as necessary. It's also helpful to cut a corner to help you orient your blot.
    a. Be very careful. Blotting films are extremely sensitive to light. If you expose an entire box of film to light, you will carve your name into the western blot hall of shame for eternity.
10. Expose blotting film to the membrane inside the film case. Generally, start with 30 second exposures, then gradually expose more if necessary.
11. After exposure, put film into developer and wait.
    a. If you have signal, yay!
    b. If you don't, increase exposure time... or you just wasted 5 days of your life.
    c. If your blotting film is pitch-black...you probably exposed it to light, or your exposure time is too long
12. When done, match up your favorite blot with the membrane. Use markers to draw the ladders.
13. Stain the membrane in Ponceau. Reuse Ponceau, rinse and dry membrane, and keep the membrane as a trophy of your hardships.

a. Be careful with Ponceau. If you accidentally get it on yourself, you'll look like you've been murdered. Side note: it smells like cherry.